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Abnormal behavior in mice mutant for the Disc1 binding partner, Dixdc1

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Disrupted-in-Schizophrenia-1 (*DISC1*) is a genetic susceptibility locus for major mental illness, including schizophrenia and depression. The Disc1 protein was recently shown to interact with the Wnt signaling protein, DIX domain containing 1 (*Dixdc1*). Both proteins participate in neural progenitor proliferation dependent on Wnt signaling, and in neural migration independently of Wnt signaling. Interestingly, their effect on neural progenitor proliferation is additive. By analogy to Disc1, mutations in *Dixdc1* may lead to abnormal behavior in mice, and to schizophrenia or depression in humans. To explore this hypothesis further, we generated mice mutant at the *Dixdc1* locus and analyzed their behavior. *Dixdc1*^{-/-} mice had normal prepulse inhibition, but displayed decreased spontaneous locomotor activity, abnormal behavior in the elevated plus maze and deficits in startle reactivity. Our results suggest that *Dixdc1*^{-/-} mice will be a useful tool to elucidate molecular pathophysiology involving Disc1 in major mental illnesses.

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Introduction

Schizophrenia is a debilitating psychiatric disorder with a worldwide lifetime risk of approximately 1%. It is thought to be caused by a combination of genetic factors and environmental insults.^{1,2} Several promising candidate susceptibility genes have been identified for schizophrenia, including *NRG1*, *DTNBP1* and *DISC1*. The dissection of molecular pathways centered on these candidate susceptibility genes is accordingly a major current priority for biological psychiatry that can lead to a better understanding of the pathogenesis in schizophrenia and possibly in other mental illnesses.

DISC1 was originally identified as a candidate susceptibility gene for major psychiatric disorders based on studies of a chromosomal translocation in a large Scottish family with a history of schizophrenia as well as major affective disorders.³ Genetic linkage studies have since implicated mutations in *DISC1* as a risk factor for schizophrenia, schizoaffective disorder, bipolar disorder, major depression and the autism spectrum disorders. Several *Disc1*-mutant mouse lines have been engineered over the last 4 years.^{4–11} All these mouse lines display behavioral abnormalities, including in the cognitive and emotional realms, supporting a role for *DISC1* mutations in the etiology of psychiatric disorders, and establishing Disc1 and the molecular pathways in which it participates as potential targets for diagnostic and therapeutic innovations.

Disc1 has multiple roles in neural development, including in morphogenesis, maturation, migration and synaptic integration of brain neurons^{12–14} through its interactions with several different protein partners.^{14–18} In a recently published study, Mao *et al.*¹⁹ discovered that among these partners is GSK3β,

a kinase central to the Wnt/β-catenin signaling pathway that has independently been suggested to contribute to psychiatric pathophysiology.^{20,21} Disc1 enhances Wnt/β-catenin-dependent neural progenitor proliferation by inhibiting GSK-3β, thereby inducing β-catenin/TCF/LEF-dependent gene transcription. More recently, another Wnt signaling pathway regulator, *Dixdc1* has been shown to form complexes with Disc1.²² *Dixdc1*, also called *Ccd1*, is a scaffold protein that possesses both Dishevelled-Axin (DIX) and coiled-coil protein-interaction domains that can act as a positive regulator of the Wnt/β-catenin pathway.²³ Concordant with the notion that *Dixdc1*, Disc1 and at least some forms of Wnt signaling cooperate in aspects of neural development relevant to psychiatric pathogenesis, knockdown of *Dixdc1* in the mouse embryonic neocortex has been reported to result in a Wnt/β-catenin-dependent reduction in neural progenitor cell proliferation, and a Wnt/β-catenin-independent inhibition in neuronal migration.²²

Given its molecular interactions with Disc1, and its role in both Wnt/β-catenin-dependent and Wnt/β-catenin-independent aspects of neural development, we hypothesized that DIXDC1 might contribute to pathophysiology underlying the abnormal behaviors in schizophrenia and other major psychiatric disorders. To explore this hypothesis further, we generated mice mutant at the *Dixdc1* locus and analyzed their behavior.

Materials and methods

Generation of *Dixdc1*-mutant mice. To create the 5'- and 3'-recombinogenic arms of a targeting construct, approximately

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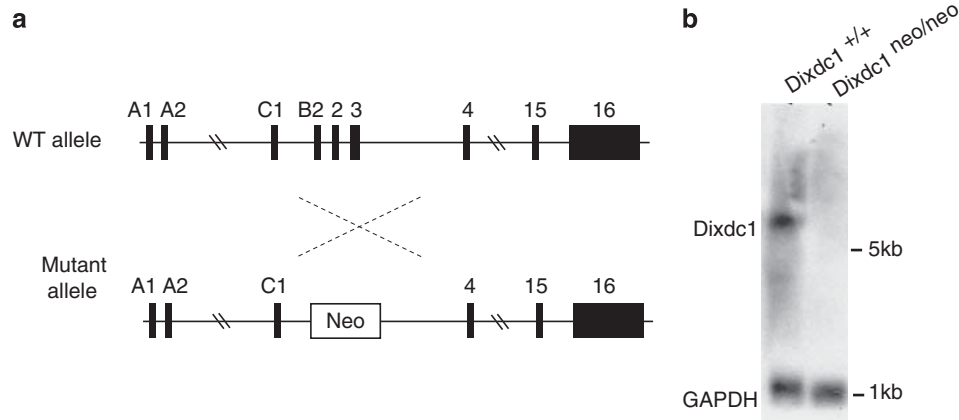


Figure 1 Generation of *Dixdc1*-mutant mice. (a) Schematic representation of *Dixdc1* gene-targeting strategy. Exons B2, 2 and 3 were replaced by a neo cassette by homologous recombination, which is predicted to introduce a premature stop codon in all *Dixdc1* transcripts. (b) Northern blot of postnatal cerebral cortical lysates using a probe complementary to most of the *Dixdc1* coding sequence (Methods). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WT, wild type.

2.4 and 3.9kb, respectively, of the *Dixdc1* locus from the 129/Sv mouse strain were inserted into pBluescript containing neomycin-positive selection and thymidine kinase-negative selection cassettes under PGK promoter control. Upon successful targeting, the neomycin expression cassette replaced exons B2, 2 and 3, and is therefore expected to disrupt all *Dixdc1* splice forms²⁴ (Figure 1). Correct targeting through homologous recombination in embryonic stem cells was confirmed by Southern blot and PCR. Mice carrying the targeted allele were generated using standard embryo manipulation and chimera breeding techniques. Genotyping of littermates from heterozygous intercrosses in the established colony was performed by genomic PCR using the following allele-specific primers: Neo-3.9: 5'-CATAGCGTTGGCTACCCGTGATATTG-3', and 3.9-Bam: 5'-GGAAGTGTGAGCTTTTCCGGGAA-3', 3.9-4: 5'-GGTGATGGATTGCGAAAGAATTCT-3'. PCR amplification was conducted for both alleles simultaneously, using all three primers in one reaction and the following parameters: 30 cycles of 94 °C, 60 s; 60 °C, 120 s and 72 °C, 60 s. The wild type allele produces a 240-bp band, whereas the targeted allele produces a 340-bp band when resolved by electrophoresis in a standard agarose gel.

Northern blot analysis. A DNA probe corresponding to exons 3–16 of *Dixdc1* was labeled with ³²P-labeled dCTP through random priming, and then purified by precipitation. 20 µg total RNA per lane, purified from cerebral cortex (postnatal day 6), was separated in 1% denaturing formaldehyde gel, blotted onto nylon membranes, and then hybridized. We used the following stringencies and times: hybridization overnight at 55 °C in UltraHyb buffer (Ambion, Austin, TX, USA), washing for 3 × 15 min in 0.2 × saline-sodium citrate buffer (SSC), 0.5% SDS (sodium dodecyl sulphate) at 68 °C. Exposure to film was overnight (15 h) at –80 °C, with two intensification screens. Transcript sizing was performed by direct comparison with Millenium marker (Ambion, Austin, TX, USA) RNA ladder.

Behavioral tests. Male mutant mice ($n=10$) and their wild type (WT) littermates ($n=9$), born to heterozygous parents,

underwent a series of behavioral tests (elevated plus maze, open field, forced swim, startle reactivity and prepulse inhibition (PPI), in that order, with a 7-day inter-test delay to allow the mice to rest) at the age of 3–4 months. For all tests, the experimenter had no knowledge of the genotype of the mice being tested and analyzed.

Elevated plus maze. The elevated plus maze is a commonly used test to assess anxiety-like behavior in rodents.^{25–27} The elevated plus maze apparatus consists of four alleys, two of which are open and the remaining two are enclosed, which intersect each other to form a cross or 'plus' (height: 41 cm, arm length: 29 cm, arm width: 9 cm). Mice were placed in the center of the maze under dim ambient light conditions and allowed to explore freely for 5 min. During this time, the mice were manually scored for the time they spent in the open arms (as defined by the placement of all four paws in one of the open arms).

Open field. Locomotor activity measurements were performed in Plexiglas locomotor activity chambers (43 × 43 cm, Med Associates, St Albans, VT, USA), located in sound-attenuating cubicles equipped with a 2.8-W house light and exhaust fans that mask the external noise. The activity chambers contained two sets of 16 pulse-modulated infrared photobeams on opposing walls to record x–y–z ambulatory movements, and were computer interfaced for data sampling at a 100-ms temporal resolution. To measure the open-field activity, mice were placed individually in the center of a chamber and tracked for 60 min. Data collected included horizontal activity (total horizontal distance, cm per 5 min), vertical activity (counts of rearing), ambulatory episodes and average speed during ambulatory episodes (cm second^{–1}).

Forced swim test. The Porsolt or 'forced' swim test is an assay of 'behavioral despair' or depressive-like behavior in rodents that has been pharmacologically validated, particularly for selective serotonin reuptake inhibitors (SSRIs).^{28,29} Swim sessions were conducted by placing each subject mouse for 6 min in individual polycarbonate cylinders (25.3 cm

tall × 22.2 cm in diameter) filled with water (23–25 °C) to a depth of 15 cm. Mice were scored manually for time immobile over this 6-min swim session. Immobility was defined as the absence of movement, except as necessary to keep afloat, including passive floating and turning in the absence of an active swimming motion. The water in the cylinder was changed for each subject. Immediately after testing, the animals were dried with a paper towel and placed in a recovery cage, half of which was positioned over an electric heat source, for a duration of 10 min before being returned to their homecage.

Acoustic startle reactivity (ASR)/ PPI. Both ASR and PPI were measured using the SR-Lab System (San Diego Instruments, San Diego, CA, USA).³⁰ During all testing, the background was set at 70-dB white noise. The ASR test consisted of eight trial types, each of which was presented five times in pseudorandom order. The trial types consisted of a single 40-ms startle stimulus of 90, 95, 100, 105, 110, 115, 120 or 125 dB. Trials were presented with a variable intertrial interval of 15–20 s. At the onset of each startle stimulus, maximum velocity (V_{max}) in arbitrary units was recorded every 1 ms for 65 ms. The V_{max} was averaged across the trial types.

The PPI test consisted of a 5-min acclimation period, followed by seven trial types presented six times each in pseudorandom order. Each trial consisted of a 20-ms prepulse, an 80-ms interval and a 40-ms startle stimulus. Prepulse intensity was set at background, 74, 78, 82, 86 or 90 dB, and the startle stimulus in each case was 120 dB. One trial type consisted solely of background noise to control for general movement of the subject. Trials were presented with a variable intertrial interval of 15–20 s. For each subject, the entire PPI test session lasted approximately 18 min. At the onset of the startle stimulus, maximum velocity (V_{max}) in arbitrary units of the startle platform was recorded every 1 ms for 65 ms. The V_{max} was averaged across trial type, with PPI calculated as the difference between V_{max} after prepulse trials (V_{max-PP}) and V_{max} after startle stimulus alone trials ($V_{max-ctl}$), and was expressed as a percentage of $V_{max-ctl}$: $PPI = ((V_{max-ctl}) - (V_{max-PP}) / (V_{max-ctl})) * 100\%$.

Results and discussion

Using homologous recombination technology, *Dixdc1*-mutant mice were genetically engineered in which exons B2, 2 and 3 were replaced by a neo cassette (Figure 1a). This mutation is predicted to introduce a premature stop codon altering all splice isoforms of *Dixdc1*.²⁴ *Dixdc1*^{neo} mice were

backcrossed for three generations to the C57BL/6 isogenic mouse strain for use in this study; *Dixdc1*^{neo/neo} mice are hereafter referred to as mutants (or $-/-$ in Figures). No *Dixdc1* RNA transcript could be detected by Northern blot of brain lysates from mutant mice, validating *Dixdc1* genetic disruption (Figure 1b). The mutants were born at Mendelian ratios, were viable and fertile, and were visually indistinguishable from their WT littermates. Gross morphology and size of the mutant neonatal brains were similar to WT, and analysis of neuronal subpopulations by *in situ* mRNA hybridization with *Dlx1*, *Tbr1*, *Tle4*, *Pbx3* and *Hef1* probes on cortical slices (embryonic day 16) did not reveal obvious differences between genotypes (data not shown).

In an open field, the mutant mice travelled significantly less distance (Figure 2a) and exhibited significantly less vertical activity counts than the WT (Figure 2b). Furthermore, the *Dixdc1*^{-/-} mice were characterized by fewer ambulatory bouts than littermate controls (Figure 2c), whereas their average speed of locomotion during ambulatory bouts was normal (Figure 2d). These data suggest that the reduced activity of *Dixdc1*^{-/-} mice is not due to a physical defect. Interestingly, decreased spontaneous locomotor activity in the open field contrasts with the hyperactivity observed in most *Disc1* mutant models.^{9–11} In the forced swim test, mutants displayed a non-significant trend towards more time immobile than WT (Figure 3a) suggesting reduced motivation. In an elevated plus maze, *Dixdc1* mutants spent significantly less time in the open arms than WT (Figure 3b),

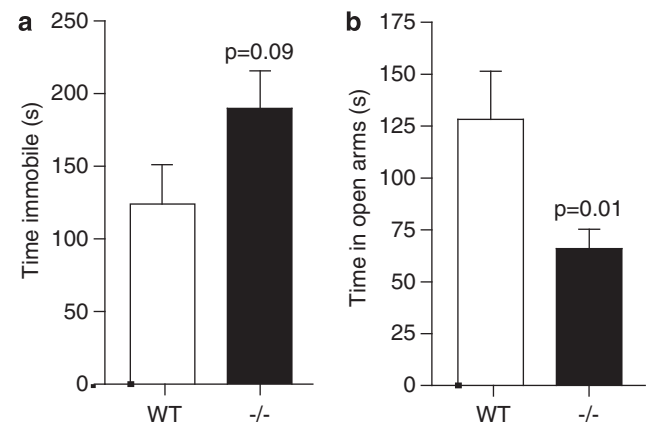


Figure 3 Motivation- and anxiety-like behavior in *Dixdc1*^{-/-} mice. (a) Time immobile in the forced swim test (Student's *t*-test). (b) Time in the open arms of the elevated plus-maze (Student's *t*-test). WT, wild type; p, probability value.

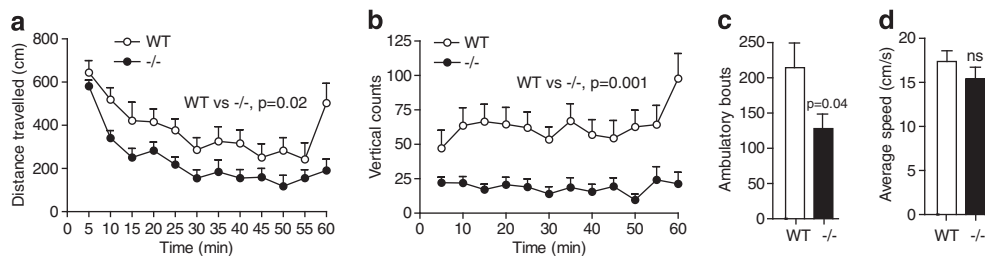


Figure 2 Spontaneous locomotion of *Dixdc1*^{-/-} mice. (a) Locomotor activity in an open field (Repeated measures two-way analysis of variance (ANOVA), wild type (WT) vs $-/-$, $F(1, 187) = 5.88$). (b) Vertical counts in an open field (Repeated measures two-way ANOVA, WT vs $-/-$, $F(1, 187) = 13.76$). (c) Number of ambulatory bouts (Student's *t*-test). (d) Average speed during ambulatory bouts (Student's *t*-test, $P = 0.28$). NS, not significant; P, probability value.

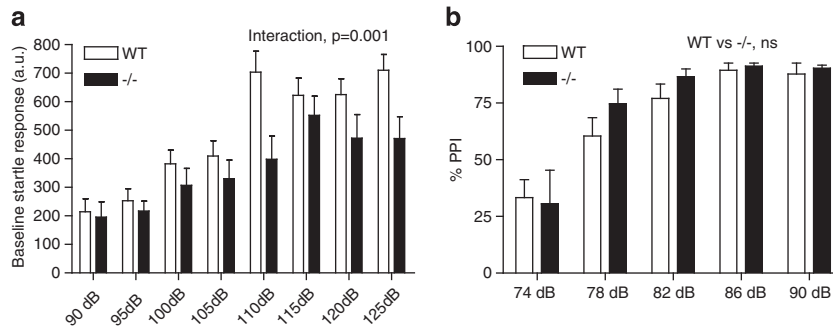


Figure 4 Baseline acoustic startle and prepulse inhibition (PPI) in *Dixdc1*^{-/-} mice. (a) Acoustic startle response (Repeated measures two-way analysis of variance (ANOVA), Interaction, $F(7, 119) = 3.60$ followed by the Bonferroni multiple comparison test, $P < 0.01$ for 110 dB) (b) PPI of the acoustic startle response (Repeated measures two-way ANOVA, wild type (WT) vs mutant, $F(1, 68) = 0.47$, $P = 0.47$). NS, not significant.

consistent with increased anxiety. However, differences observed in both the forced swim test and the elevated plus maze may be confounded by the generally reduced locomotor activity documented in the open-field test. Resolution of this issue awaits more comprehensive behavioral phenotyping and analyses.

Similar to some *Disc1* mutant models,¹¹ the ASR in *Dixdc1* mutants was abnormal; startle responses to tones ≥ 110 dB were decreased (significant difference at 110 dB only), whereas responses to tones < 110 dB were similar to the WT (Figure 4a). Nonetheless, in contrast to some *Disc1*-mutant models and to other mouse models for schizophrenia,^{4,10,11,31} the PPI of the startle response was no different from WT in *Dixdc1* mutants (Figure 4b).

To summarize, we have engineered a line of mice, which lacks *Dixdc1*, a partner of the *Disc1* protein implicated in schizophrenia. *Dixdc1*-mutant mice are viable, fertile, have grossly normal brains at birth and display normal PPI, but have decreased spontaneous locomotor activity in an open field, abnormal behavior in the elevated plus maze and deficits in startle reactivity—a complex behavioral phenotype that though not immediately relatable to a specific psychiatric disorder nonetheless supports a role for this gene along with *DISC1* in general psychiatric pathophysiology. One caveat to the present study is that the *Dixdc1*^{neo} mice studied here were backcrossed only for three generations to the C57BL/6 isogenic mouse strain, whereas backcrosses for 10 generations or more are recommended to homogenize genetic background in comparative behavioral studies. Increasing the number of backcrosses would also reduce the chances of phenotypic effects arising from allelic differences at co-segregating flanking genes derived from the 129/Sv embryonic stem cell strain used for *Dixdc1* gene targeting. In summary, further behavioral analyses in isogenic mice will be required to fully characterize the spectrum of sensory, affective and cognitive deficits in *Dixdc1*-mutants, but the present study highlights the potential utility of this novel mouse strain in elucidating roles of this gene, its partner *Disc1* and related molecular pathways in the pathogenesis of behavioral disorders.

Conflict of interest

The authors declare no conflict of interest.

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